

CSP α reduces aggregates and rescues striatal dopamine release in α synuclein transgenic mice

Caló L^{1,2}, Hidari E^{2,3}, Wegrzynowicz M¹, Dalley JW^{4,5}, Schneider BL^{6,7}, Anichtchik O¹, Carlson E¹, Klenerman D^{2,3}, Spillantini MG¹

¹Department of Clinical Neurosciences, Clifford Allbutt Building, University of Cambridge, Cambridge, UK. ²Dementia Research Institute, University of Cambridge, Cambridge, UK. ³Department of Chemistry, University of Cambridge, Cambridge, UK. ⁴Department of Psychology, University of Cambridge, Cambridge, UK. ⁵Department of Psychiatry, Hershel Smith Building for Brain and Mind Sciences, University of Cambridge, Cambridge, UK. ⁶Brain Mind Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland. ⁷Bertarelli Platform for Gene Therapy, Ecole Polytechnique Fédérale de Lausanne (EPFL), Geneva, Switzerland.

Correspondence to:

Prof Maria Grazia Spillantini and Dr Laura Caló

Department of Clinical Neurosciences,

Clifford Allbutt Building

Hills Road, Cambridge CB2 0AH, UK

E-mails: mgs11@cam.ac.uk; lc232@cam.ac.uk

Abstract

α Synuclein aggregation at the synapse is an early event in Parkinson's disease and is associated with impaired striatal synaptic function and dopaminergic neuronal death. The cysteine string protein (CSP α) and α synuclein have partially overlapping roles in maintaining synaptic function and mutations in each cause neurodegenerative diseases. CSP α is a member of the DNAJ/HSP40 family of co-chaperones and like α synuclein, chaperones the SNARE complex assembly and neurotransmitter release. α Synuclein can rescue neurodegeneration in CSP α KO mice. However, whether α synuclein aggregation alters CSP α expression and function is unknown. Here we show that α synuclein aggregation at the synapse induces a decrease in synaptic CSP α and a reduction in the complexes that CSP α forms with HSC70 and STGa. We further show that viral delivery of CSP α rescues *in vitro* the impaired vesicle recycling in PC12 cells with α synuclein aggregates and *in vivo* reduces synaptic α synuclein aggregates restoring normal dopamine release in 1-120h α syn mice. These novel findings reveal a mechanism by which α synuclein aggregation alters CSP α at the synapse, and show that CSP α rescues α synuclein aggregation-related phenotype in 1-120h α syn mice similar to the effect of α synuclein in CSP α KO mice. These results implicate CSP α as a potential therapeutic target for the treatment of early-stage PD.

Key words: α -Synuclein, CSP α , Parkinson's disease, transgenic mice, synapse, dopamine, DNAJ chaperon family.

Abbreviations: CSP α = cysteine string protein; KO = knock-out; 1-120h α Syn =1-120 truncated human α synuclein; PD = Parkinson's disease; SNARE = soluble N-ethylmaleimide sensitive fusion attachment protein receptor

Introduction

Alpha-synuclein (α syn) is a synaptic protein involved in vesicle clustering, assembly of the SNARE complex and neurotransmitter release. Point mutations and duplication/triplication of the α synuclein gene cause Parkinson's disease (PD) (Lunati *et al.*, 2018) and α syn aggregates form the Lewy bodies characteristic of PD (Spillantini *et al.*, 1998, 1997). C-terminal truncation of α syn, found in Lewy bodies, promotes its aggregation (Baba *et al.*, 1998; Crowther *et al.*, 1998). We previously described 1-120h α syn transgenic mice expressing C-terminally truncated α syn under the control of the tyrosine hydroxylase-promoter in the absence of the endogenous protein (Tofaris *et al.*, 2006), where α syn aggregation in the striatal terminals is associated with re-distribution of SNARE proteins and impairment in dopamine (DA) release, features present in PD patients (Garcia-Reitböck *et al.*, 2010). Growing evidence points to presynaptic terminals as the initial site of neurodegeneration in PD (Nakata *et al.*, 2012; Janežic *et al.*, 2013; Garcia-Reitböck *et al.*, 2010; Wegrzynowicz *et al.*, 2019), as shown in our transgenic MI2 mice, where synaptic dysfunction with α syn accumulation preceded DA cell death, both rescued by an oligomer modifier (Wegrzynowicz *et al.*, 2019).

The cysteine string protein α (CSP α /DNAJC5) is a vesicle-associated protein that regulates neurotransmitter release, exocytosis/endocytosis coupling and SNARE complex assembly through a pathway parallel to that of α syn (Tobaben *et al.*, 2001; Chandra *et al.*, 2005; Zhang *et al.*, 2012), DNAJC proteins have been linked to parkinsonism (Roosen *et al.* 2019). CSP α function is mediated by the DNAJ domain which activates the ATPase activity of the Heat shock cognate 70kDa protein HSC70 (Braun *et al.*, 1996; Chamberlain *et al.*, 1997); its mechanism of action is functionally associated with α syn in that overexpression of α syn abolishes lethal neurodegeneration in CSP α -KO mice and ablation of all three (α , β , γ)-syn genes results in SNARE complex assembly deficit with an increase in CSP α (Burre *et al.*, 2010; Goremberg *et al.*, 2017). However, whether CSP α levels and activity change with α syn aggregation and SNARE protein redistribution or if CSP α can rescue the synaptic pathology associated with α syn aggregation is not known.

In this study we show that CSP α expression and function are impaired in the presynaptic terminals of 1-120h α syn mice concomitantly with the presence of α syn aggregates and a reduction in evoked DA release. We further show that expression of CSP α rescues both α syn

-aggregation dependent deficit in vesicle cycle *in vitro* and impaired DA release *in vivo*. This effect is associated *in vivo* with reduction in the number of striatal synaptic α syn aggregates as shown by super resolution analysis of tissue sections.

Materials and Methods

Mice

Transgenic 1-120h α syn and control mice without endogenous α syn (C57BL/6/OlaHsd) were used in this study (Tofaris *et al.*, 2006; Garcia-Reitböck *et al.*, 2010).

Regulated animal procedure were carried out under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB), under project license no. 7008383.

Immunostaining

Brains from paraformaldehyde-perfused 12 month-old mice were sectioned and 30 μ m free-floating sections were incubated overnight at 4°C with primary antibodies (anti- α syn, BD Transduction, 1:700), anti-DNAJC5 (Millipore, 1:500) as previously done (Garcia-Reitböck *et al.*, 2010). Staining was visualised using the ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine or Alexa-labelled secondary antibodies and imaged using a Leitz DMRB microscope or a Leica TCS SPE confocal microscope.

Co-immunoprecipitation and Immunoblotting

Total proteins were extracted from mouse striata in PBS containing 0.1% Tween 20 and Protease Inhibitor cocktail (Roche) with or without 1 mM non-hydrolysable ADP (Sigma). Immunoprecipitation followed previous protocols (Garcia-Reitböck *et al.*, 2010). Briefly, proteins (0.8-1 mg) were rotated overnight at 4°C with 5 μ g of mouse anti-SGTa antibody (Abcam) or control mouse IgG and protein G Dynabeads (Invitrogen). Immunocomplexes were eluted by denaturation in NuPAGE LDS sample buffer (Invitrogen). Synaptosomal fractions were extracted using Syn-PER synaptic protein extraction reagent (Thermo Scientific). Proteins were resolved on a 4-12% gradient PAGE-SDS gel (Invitrogen), transferred onto nitrocellulose membranes (Bio-Rad), incubated with peroxidase-conjugated secondary antibodies (GE Healthcare) and visualised with chemiluminescent substrates (Thermo Fisher Scientific) as previously described (Wegrzynowicz *et al.*, 2019). Antibodies

were: mouse anti-DNAJC5 (Millipore 1:500), anti-VAMP2 (Abcam 1:500), anti-ATPase HSC70 (Synaptic systems, 1:500), anti-SGTa (Abcam 1:500) and rabbit anti- β -actin (Abcam, 1:10000).

AAV vector injections

Human full-length CSP α cDNA, a gift from Prof RD Burgoyne (Liverpool University), was subcloned into a pAAV vector under the PGK promoter and packaged in serotype 6 AAV particles as described (L \ddot{o} w *et al.*, 2013). Vector suspension was diluted to 1×10^{13} viral genome containing particles (VG)/mL and an AAV6 empty vector (EV) used as control. For vector injections, animals were anesthetized with 2% isoflurane, placed in a stereotaxic frame (David Kopf Instruments) and injected bilaterally with 2 μ l of the virus (0.2 μ l/min flow rate) in the substantia nigra (SN) at the following coordinates: AP=+0.7, L= \pm 1.7, DV=-3.6 below dural surface relative to the bregma according to Paxinos and Watson (Paxinos and Franklin, 2004).

Retention of FM1-43

PC12 cells stably expressing 1-120h α Syn were plated onto coverslips in 12 well plates at 7×10^5 cells/ml (Garcia-Reitbock *et al.*, 2010). Cells were infected with 1 μ l 1×10^{13} VG/mL AAV6CSP α or control AAV6EV for 24 hours, then grown for 4 days in fresh medium. To stimulate vesicle endocytosis of the FM1-43 dye (Invitrogen), PC12 cells were depolarised with KCl (Hank's balanced salts medium with Ca²⁺ and Mg²⁺, 90 mM KCl, 63 mM NaCl) then incubated with 15 μ M FM1-43 for 90 s at room temperature and unbound dye removed by 10 min wash in PBS /1mM scavenger dye ADVASEP-7 (Sigma). Cells were re-incubated with depolarizing solution for 90 s at room temperature (Gaffield *et al.*, 2006; Garcia-Reitbock *et al.*, 2010). Only vesicles with impaired release retained the dye. Cells were then washed, fixed with 4% paraformaldehyde and stained (Syn1 antibody, BD Biosciences, 1:500) overnight at 4°C. Signal was detected using a Leica SPE 4 confocal microscope. FM1-43 positive puncta above the threshold fluorescence set by the AAV6EV transduced cells were counted using ImageJ analysis software. Between 700-1000 cells were counted for each experimental condition.

***In vivo* microdialysis**

In vivo microdialysis was performed as previously reported (Garcia-Reitbock *et al.*, 2010; Wegrzynowicz *et al.*, 2019). A microdialysis cannula (CMA Microdialysis) was placed in

anesthetized mice in the right medial striatum (AP = +0.7, L = +1.7, H = -2.1 from the bone [31], DV = -2.1 from the skull surface). The following day, a CMA/7 microdialysis probe was inserted into the guide cannula and perfusion performed at a constant flow rate (2 μ l/min) with artificial cerebrospinal fluid (ACSF: 140 mM NaCl, 7.4 mM glucose, 3 mM KCl, 0.5 mM MgCl₂, 1.2 mM CaCl₂, 1.2 mM Na₂HPO₄, 0.3 mM NaH₂PO₄, pH 7.4). Dialysates were collected every 20 min in tubes containing 5 μ l of 0.2 M perchloric acid to prevent dopamine oxidation and assayed for dopamine, homovanillic acid and 3,4-dihydroxyphenylacetic acid. Two fractions (20-40 min) were collected to evaluate baseline release, ACSF was then replaced by ACSF containing 50 mM KCl and three more fractions collected (60-100 min). High KCl ACSF was then replaced by basal ACSF and two more fractions (120-140 min) were collected after which mice were killed, and brains used for immunohistochemistry or immunoblotting. Dopamine and homovanillic acid levels in the dialysate were measured by high-performance liquid chromatography (Garcia-Reitböck *et al.*, 2010; Wegrzynowicz *et al.*, 2019).

dSTORM

Thirty-micron free-floating striatal sections were stained for α syn (Syn1, BD, 1:300) and Alexa Fluor Plus 647 secondary antibody (Invitrogen, 1:2000) in the presence of Tetra Speck microspheres (Invitrogen) to correct for drift during imaging.

Images were acquired with Photometrics EMCCD camera on a Nikon Ti-2E inverted microscope in near-TIRF mode. Image stacks consisted of 10,000 frames (50 ms/frame) on the field of view (FOV). A total of four-five FOVs per section and four mice per group were tested.

The image stacks were drift corrected and analysed using PeakFit in the open source ImageJ plugin GDSC SMLM, followed by a custom script. Briefly, this grouped the fluorescence signals into clusters (monomers or aggregates) based on their spatiotemporal distribution to determine their area. The aggregate size reported is the square root of the area (Whiten *et al.*, 2018,*b*).

Experimental design and statistical analysis

Immunoblotting: relative band intensity (RI) was calculated using Image J and β Actin-normalised CSP α , HSC70, STGa and VAMP2 levels analysed with two-tailed Student's t test.

Co-immunoprecipitation: RI of CSP α , HSC70, and SGTa alongside corresponding input proteins were normalised to β Actin and analysed using one-way ANOVA with Bonferroni's multiple comparisons test.

FM1-43 dye retention: data were evaluated using two-way ANOVA with Bonferroni's multiple comparison test.

Microdialysis: DA release was normalized to the baseline fraction (0 min) and expressed as fold difference relative to the average DA release directly following K⁺ stimulation (60 min fraction) in the control group. DA release in 1-120h α Syn or control mice treated with CSP α or EV was analysed using two-way ANOVA with Bonferroni's multiple comparison test.

dSTORM: species were divided into monomer/aggregates based on their size with recombinant monomers having a median length < 36.5 nm (20.65 \pm 2 \times 7.9 mean+2 \times SD, Wegrzynowicz *et al.*, 2019). Data were analysed using the software https://github.com/Eric-Kobayashi/SR_toolkit.

Median size was analysed using a two-tailed Student's t test; size distribution differences between CSP α and EV-treated groups (monomers and aggregates combined) was calculated from a cumulative histogram using a Kolmogorov-Smirnov test.

Results

CSP α levels are altered in the striatum of 1-120h α Syn mice

α Syn aggregates were present in the striatum of 1-120h α Syn 12 month-old mice, no α syn staining was present in background control mice lacking the endogenous protein (Tofaris *et al.*, 2006; Garcia-Reitböck *et al.*, 2010). CSP α staining in the striatum of control mice revealed a punctate pattern that was less intense in 1-120h α Syn mice (Fig 1A). By immunoblotting no significant difference was present in CSP α amounts between controls and 1-120h α Syn mice in total tissue homogenates, however, a significant 68.3% reduction in CSP α amount was present in synaptosomal protein extracts in 1-120h α Syn mice (Fig. 1B) (Total homogenates RI: Control 1.29 \pm 0.26; 1-120h α Syn 1.07 \pm 0.27; Synaptic fraction: control 1.83 \pm 0.21; 1-120h α Syn 0.58 \pm 0.16). HSC70 and SGTa were not changed in the synaptic fraction (Supplementary Fig. 1). The level of the synaptic v-SNARE protein VAMP2 was also not changed as previously reported (Garcia-Reitböck *et al.*, 2010; Supplementary Fig. 1).

CSP α in synaptic terminals forms a trimeric complex with HSC70 and SGTa. To test whether this function was perturbed in the striatum of 1-120h α Syn mice, we used immunoprecipitation in the presence of ADP. We found a reduction in the amount of CSP α and HSC70 complexes in 1-120h α Syn mice compared to controls. No changes in SGTa, HSC70 and CSP α expression levels were found in the total homogenate input (Fig. 1C) (RI: HSC70: control 93031 \pm 3690, 1-120h α Syn 53078 \pm 1726; CSP α : control 69117 \pm 3181, 1-120h α Syn 18097 \pm 2762; SGTa: control 87661 \pm 5166, 1-120h α Syn 81385 \pm 4499). Taken together, these findings suggest that the presence of aggregated α syn in 1-120h α Syn mice selectively reduces CSP α levels in striatal synaptic terminals hampering its chaperone activity.

CSP α rescues vesicle cycling impairment in PC12 cells expressing 1-120h α Syn

As previously reported expression of 1-120h α Syn in PC12 cells alters vesicle endo/exocytosis (Garcia-Reitböck *et al.*, 2010). To determine whether CSP α could rescue the α syn-related vesicle turnover impairment, PC12 cells stably expressing 1-120h α Syn were transduced with either an AAV6 vector encoding human CSP α (AAVCSP α), or an empty AAV6 control vector (AAVEV). In a control experiment, non-transfected PC12 cells were also treated with AAVCSP α or EV (Fig. 2).

A cycle of vesicle uptake and release was induced by K⁺ in the presence of FM1-43 dye and the number of cells that internalised and retained the dye above a threshold value of fluorescence was counted. Fluorescence was increased in cells stably expressing 1-120h α Syn following K⁺ treatment, compared to un-transfected cells (endo) as previously shown (Garcia-Reitböck *et al.*, 2010). Expression of CSP α in 1-120h α Syn expressing cells reduced the number of cells that retained FM1-43 while no effect was observed in cells treated with EV. Furthermore, CSP α or EV viral expression did not alter the release of FM1-43 from wild-type cells expressing endogenous α syn (Fig. 2B) (cell number: 1-120h α syn: EV =55 \pm 4.9, CSP α =16.6 \pm 4.6; endo: EV 16.9 \pm 3.6, CSP α 17.9 \pm 3). Thus, CSP α overexpression selectively rescues the synaptic vesicle cycle impairment caused by the expression of human truncated α syn.

CSP α delivery *in vivo* restores DA release

To investigate whether the effect of CSP α observed *in vitro* was present *in vivo*, we injected AAVCSP α and AAVEV into the SN of transgenic 1-120h α Syn mice which have a decrease

in striatal DA release (Garcia-Reitböck *et al.*, 2010) We first investigated the time-course of CSP α expression after injection in the SN of control mice with AAVCSP α and AAVEV and measured CSP α protein levels by immunoblotting in the striatum at 4, 6- and 8-weeks post-injection. A two-fold increase in CSP α expression in the striatum was present up to eight weeks post-injection (4 weeks: CSP α = 1.36 \pm 0.19, EV= 0.71 \pm 0.13, 6 weeks: CSP α = 1.25 \pm 0.08, EV= 0.46 \pm 0.04; 8 weeks: CSP α = 1.22 \pm 0.02, EV= 0.60 \pm 0.08) (Supplementary Fig. 2). Therefore, AAVCSP α or AAVEV were injected in the SN of 1-120h α Syn and control 10 month-old mice and the striatal DA release was measured at eight weeks post-injection using *in vivo* microdialysis.

As previously shown, K⁺-evoked DA release was significantly reduced in untreated 1-120h α Syn mice compared to controls (Garcia-Reitböck *et al.*, 2010) (fractions: 60, 80, 100 min normalised to peak control mice value (60 min); 0.47 \pm 0.045 vs 1 \pm 0.03 (60 min), 0.21 \pm 0.03 vs 0.63 \pm 0.18 (80 min), 0.12 \pm 0.03 vs 0.42 \pm 0.18 (100 min). Notably, expression of CSP α restored DA release levels in 1-120h α Syn mice back to the control values (0.98 \pm 0.06 fraction 60 min, 0.77 \pm 0.08 fraction 80 min) whereas the EV vector was ineffective (0.46 \pm 0.05 fraction 60 min, 0.29 \pm 0.05 fraction 80 min) (Fig. 3).

The effect of CSP α was dependent on α syn aggregation, because no difference was observed in control mice after treatment with CSP α or EV. (CSP α : 0.96 \pm 0.15 vs EV: 0.98 \pm 0.15 fraction 60 min, CSP α : 0.67 \pm 0.14 vs EV: 0.69 \pm 0.1 fraction 80 min) (Fig. 3).

These results show that increased expression of CSP α *in vivo* specifically rescues impaired DA release associated with 1-120 α syn aggregation.

CSP α reduces α syn aggregates and increases α syn monomers in the striatum of 1-120 h α Syn mice

To investigate whether CSP α increase had an effect on aggregation in 1-120h α Syn mice, immunostaining for α syn was performed in striata of 12 months-old mice after microdialysis. Striata from EV-treated mice showed intensely stained α syn aggregates in the neuropil, while the staining was reduced in CSP α -treated mice (Fig. 4A). To further characterise the α syn species in CSP α animals, we used *d*STORM and compared the number of aggregated versus monomeric α syn species at the nanoscopic level in both CSP α and EV-injected mice. In CSP α -injected mice the number of α syn aggregates was reduced compared to EV-treated

mice (aggregate number relative units (RU)=% of all species that are aggregated: CSP α = 66 \pm 2%, EV=78 \pm 2%) (Fig 4B).

We then analysed the difference in aggregate size distribution between EV- and CSP α -injected mice. No difference in the median size of α syn aggregates between the CSP α -injected and EV injected group was found, however CSP α -injected mice exhibited a significant 16.6% reduction in α syn aggregates (>36.5 nm) and conversely, an increase in monomeric species (<36.5 nm) (Fig 4C, D and Table) indicating that CSP α reduced the number of α syn aggregates and increased the number of α syn monomeric species.

Discussion

α Syn is predominantly localised at the synapse where it is involved in SNARE complex assembly and synaptic vesicle turnover (Iwai *et al.*, 1995; Nemani *et al.*, 2010; Burre *et al.*, 2010, 2014; Diao *et al.*, 2013; Vargas *et al.*, 2017; Longhena *et al.* 2019). CSP α is also at the synapse where it assists folding of client proteins involved in neurotransmitter release, exo/endocytosis and SNARE complex formation (Tobaben *et al.*, 2001; Chandra *et al.*, 2005; Zhang *et al.*, 2012). α Syn is functionally interconnected with CSP α as shown by its ability to rescue the neurodegeneration characteristic of CSP α -KO mice (Chandra *et al.*, 2005) but whether CSP α is affected by α syn aggregation is not clear. Here we show that CSP α immunoreactivity is reduced in the striatum of 12 month-old 1-120h α Syn mice compared to controls. This decrease was specifically in the synaptosomal fraction indicating that CSP α reduction is localised where α syn aggregates. As a synaptic co-chaperone, CSP α binds to HSC70 and the adapter protein SGTa to regulate vesicle fusion. By co-immunoprecipitation we found that CSP α /HSC70 complexes with SGTa are reduced in the striatum, effect that may be attributed to CSP α reduction. These findings indicate that α syn synaptic aggregation affects CSP α levels and function. In order to determine whether an increase of CSP α could be beneficial and restore vesicle cycle and DA release, we tested this first in PC12 cells expressing 1-120h α syn and found that indeed their abnormal vesicle cycle was restored. This prompted us to investigate whether an increase of CSP α *in vivo* in the striatum of 1-120h α Syn mice could restore DA release. Using microdialysis we found that CSP α expression restored the striatal DA release reduced by synaptic α syn aggregation, and dSTORM super-resolution microscopy performed in tissue after microdialysis, indicated a reduction in the number of α syn aggregates and a concomitant increase in monomeric

species. It is unclear whether CSP α prevents monomeric α syn aggregation or contributes to dissociation of the α syn aggregates. Both HSC70 small chaperones and other DNAJs have been shown to affect α syn aggregation (Pemberton *et al.*, 2011; Whiten *et al.*, 2018,a), in our model, CSP α could contribute to facilitate HSC70 ATPase activity as the burden of misfolded α syn increases. Although it cannot be excluded that the increase in CSP α per se could improve dopamine release, the fact that no change was observed in control mice would support that CSP α acts by affecting α syn-related alterations. The increase in monomeric α syn is similar to what we observed in MI2 transgenic mice after anle138b treatment (Wegrzynowicz *et al.*, 2019), whether CSP α beneficial effect is due the reduction of aggregates or the increase of monomeric α syn remains to be determined.

In conclusion, our data reveal for the first time that α syn aggregation impairs expression of synaptic CSP α and formation of its functional complexes with HSC70 and that increase of CSP α reduces synaptic α syn aggregates and increases monomeric α syn rescuing DA release impairment. These results point to CSP α and related chaperones as therapeutic avenues for restoring normal synaptic function in early PD.

Acknowledgements

We wish to thank Prof RD Burgoyne for the CSP α cDNA, Dr E. Dimou and Dr J. McColl for advice on *d*STORM imaging and Dr MP Fransen and Dr G Vivacqua for critically reading the manuscript.

Funding

This work was supported by the MJ Fox Foundation, the Cure PD trust, Parkinson's UK and the UK Dementia Research Institute.

Conflict of Interest

The authors have no competing financial interest.

References

- Baba M, Nakajo S, Tu PH, Tomita T, Nakaya K, Lee VM, Trojanowski JQ, Iwatsubo T. Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am J Pathol* 1998; **152**: 879-884.
- Braun JE, Wilbanks SM, Scheller RH. The cysteine string secretory vesicle protein activates Hsc70 ATPase. *J Biol Chem* 1996; **271**: 25989-25993.
- Burré J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Sudhof TC. Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science* 2010; **329**: 663-1667.
- Burré J, Sharma M, Südhof TC. α -Synuclein assembles into higher-order multimers upon membrane binding to promote SNARE complex formation. *Proc Natl Acad Sci USA* 2014; **11**: 4274-83.
- Chamberlain LH, Burgoyne RD. Activation of the ATPase activity of heat shock proteins Hsc70/Hsp70 by cysteine-string protein. *Biochem J* 1997; **322** : 853-858.
- Chandra S, Gallardo G, Fernandez-Chacon R, Schluter OM, Sudhof TC. Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. *Cell* 2005; **123**: 383-396.
- Crowther RA, Jakes R, Spillantini MG, Goedert M. Synthetic filaments assembled from C-terminally truncated alpha-synuclein. *FEBS Lett* 1998; **436**: 309-312.
- Diao J, Burre J, Vivona S, Cipriano DJ, Sharma M, Kyoung M, Sudhof TC, Brunger AT. Native alpha-synuclein induces clustering of synaptic-vesicle mimics via binding to phospholipids and synaptobrevin-2/VAMP2. *Elife* 2013; **2**: e00592.
- Gaffield MA, Betz WJ. Imaging synaptic vesicle exocytosis and endocytosis with FM dyes. *Nat Protoc* 2006; **1**: 2916-2921.
- Garcia-Reitböck P, Anichtchik O, Bellucci A, Iovino M, Ballini C, Fineberg E, Ghetti B, Della Corte L, Spano P, Tofaris GK, Goedert M, Spillantini MG. SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease. *Brain* 2010; **133**: 2032-2044.

Gorenberg EL, Chandra SS. The Role of Co-chaperones in Synaptic Proteostasis and Neurodegenerative Disease. *Front Neurosci* 2017; **11**:248.

Longhena F, Faustini G, Spillantini MG, Bellucci A. Living in Promiscuity: The Multiple Partners of Alpha-Synuclein at the Synapse in Physiology and Pathology. *Int J Mol Sci*. 2019; 20:141.

Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva H A, Kittel A, Saitoh T. The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 1995; **14**: 467-475.

Janezic S, Threlfell S, Dodson PD, Dowie MJ, Taylor TN, Potgieter D, Parkkinen L, Senior SL, Anwar S, Ryan B, Deltheil T, Kosillo P, Cioroch M, Wagner K, Ansorge O, Bannerman DM, Bolam JP, Magill PJ, Cragg SJ, Wade-Martins R. Deficits in dopaminergic transmission precede neuron loss and dysfunction in a new Parkinson model. *Proc Natl Acad Sci U S A* 2013; **110**: E4016-4025.

Löw K, Aebischer P, Schneider BL. Direct and retrograde transduction of nigral neurons with AAV6, 8, and 9 and intraneuronal persistence of viral particles. *Hum Gene Ther* 2013; **24**: 613-29.

Lunati A, Lesage S, Brice A. The genetic landscape of Parkinson's disease. *Rev Neurol* 2018;174(9):628-643.

Nakata Y, Yasuda T, Fukaya M, Yamamori S, Itakura M, Nihira T, Hayakawa H, Kawanami A, Kataoka M, Nagai M, Sakagami H, Takahashi M, Mizuno Y, Mochizuki H. Accumulation of alpha-synuclein triggered by presynaptic dysfunction. *J Neurosci* 2012; **32**:17186-17196.

Nemani VM, Lu W, Berge V, Nakamura K, Onoa B, Lee MK, Chaudhry FA, Nicoll RA, Edwards RH. Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle recluster after endocytosis. *Neuron* 2010; **65**: 66-79.

Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates. Elsevier Academic Press, Amsterdam 2004.

Pemberton S, Madiona K, Pieri L, Kabani M, Bousset L, Melki R. Hsc70 protein interaction with soluble and fibrillar alpha-synuclein. *J Biol Chem* 2011; **286**: 34690-9.

Roosen DA, Blauwendraat C, Cookson MR, Lewis PA. DNAJC proteins and pathways to parkinsonism. *FEBS J.* 2019; 286:3080-3094.

Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. Alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc Natl Acad Sci U S A* 1998; **95**: 6469-6473.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature* 1997; **388**: 839-840.

Tobaben S, Thakur P, Fernandez-Chacon R, Sudhof TC, Rettig J, Stahl B. A trimeric protein complex functions as a synaptic chaperone machine. *Neuron* 2001; **31**: 987-999.

Tofaris GK, Garcia Reitböck P, Humby T, Lambourne SL, O'Connell M, Ghetti B, Gossage H, Emson PC, Wilkinson LS, Goedert M, Spillantini MG. Pathological changes in dopaminergic nerve cells of the substantia nigra and olfactory bulb in mice transgenic for truncated human alpha-synuclein(1-120): implications for Lewy body disorders. *J Neurosci* 2006; **26**: 3942-3950.

Vargas KJ, Schrod N, Davis T, Fernandez-Busnadiego R, Taguchi YV, Laugks U, Lucic V, Chandra SS. Synucleins Have Multiple Effects on Presynaptic Architecture. *Cell Rep* 2017; **18**:161-173.

Wegrzynowicz M, Bar-On D, Calo L, Anichtchik O, Iovino M, Xia J, Ryazanov S, Leonov A, Giese A, Dalley JW, Griesinger C, Ashery U, Spillantini MG. Depopulation of dense α -synuclein aggregates is associated with rescue of dopamine neuron dysfunction and death in a new Parkinson's disease model. *Acta Neuropathol* 2019; **138**:575-595.

Whiten DR, Cox D, Horrocks MH, Taylor CG, De S, Flagmeier P, Tosatto L, Kumita JR, Ecroyd H, Dobson CM, Klenerman D, Wilson MR. Single-Molecule Characterization of the Interactions between Extracellular Chaperones and Toxic α -Synuclein Oligomers. *Cell Rep* 2018a; **23(12)**:3492-3500.

Whiten DR, Zuo Y, Calo L, Choi ML, De S, Flagmeier P, Wirthensohn DC, Kundel F, Ranasinghe RT, Sanchez SE, Athauda D, Lee SF, Dobson CM, Gandhi S, Spillantini MG, Klenerman D, Horrocks MH. Nanoscopic Characterisation of Individual Endogenous Protein Aggregates in Human Neuronal Cells. *Chembiochem* 2018b; **19**:2033-2038.

Zhang YQ, Henderson MX, Colangelo CM, Ginsberg SD, Bruce C, Wu T, Chandra SS. Identification of CSP α clients reveals a role in dynamin 1 regulation. *Neuron* 2012; **74**: 136-150.

Figure Legends

Figure 1 CSP α expression is altered in the striatum of 12 month-old 1-120h α Syn mice

A CSP α staining in striatal sections from controls and 1-120h α Syn mice. Note the loss of puncta intensity of CSP α in 1-120h α Syn mice compared to controls. Scale bar: 20 μ m. **B** Left panel: Immunoblot for anti-CSP α and β Actin (β Act) in sequentially-extracted total homogenates and synaptic fractions from striata of control and 1-120h α Syn mice. Right panel: RI of CSP α levels normalised to β actin in total homogenates and synaptic fractions. Data are presented as mean \pm SEM of N=3 mice, **P<0.01 (Student's t-Test). **C** Left panel; immunoblots of HSC70, CSP α , SGTa after co-immunoprecipitation with anti-SGTa antibody with non-hydrolysable ADP. CSP α and HSC70 complexes with SGTa are reduced in 1-120h α Syn mice compared to controls whereas input levels do not change (lower panel, CSP α , HSC70 and SGTa and correspondent β Actin). Right panel; RI of HSC70, CSP α , SGTa relative to their input levels (total homogenates prior co-immunoprecipitation). Values in graph represent N=4-5 mice in 3 independent experiments ***P<0.001 (One-way ANOVA with Bonferroni's multiple comparison test).

Figure 2 CSP α rescues the vesicle cycle impairment in PC12 cells expressing 1-120h α Syn

A FM1-43 dye fluorescence (left panels, green) and α syn staining (right panels, red) in cells treated with empty vector (EV) or CSP α . Note the reduction in FM1-43 dye retention in cells stably expressing 1-120h α syn treated with CSP α compared to their EV-treated counterpart (arrows). Treatment with CSP α or EV had no effect in non-transfected PC12 cells (endo). **B** Quantification of the number of cells that retained FM1-43 dye above basal levels in every treatment group. Values are mean \pm SEM of N=12 independent experiments ***P<0.001, Two-Way ANOVA Bonferroni's multiple comparison test. Scale bar 10 μ m.

Figure 3 CSP α restore DA release impairment in 1-120h α syn mice

Striatal DA release after infusion of 50mM KCl for 60 min during *in vivo* microdialysis. Twelve month-old 1-120h α Syn mice showed a significant reduction in DA release following KCl stimulation compared with controls (*** P<0.001, *P<0.05). Values show fold change compared to baseline fractions.

Increase of CSP α following AAVCSP α injection restored DA release to control levels in 1-120h α Syn mice whereas treatment with AAVEV was ineffective (fractions: 60 min ## P<0.01, 80 min # P<0.05). No significant difference was observed in the 60- and 80-min fractions between CSP α and EV-treated or untreated control mice. Values are mean \pm SEM of N=5-7 1-120h α Syn and N=5-6 control mice per treatment group, two-way ANOVA, Bonferroni's multiple comparison test.

Figure 4 CSP α reduces α syn aggregates and increases α syn monomers in the striatum of 1-120h α Syn mice

A α Syn immunostaining in the striata of CSP α and EV treated 1-120h α syn mice. Scale bar 10 μ m. **B** Aggregate number (RU) versus monomeric α syn in AAVEV (EV) and AAVCSP α (CSP α)-treated mice based on *d*STORM analysis using anti- α syn Syn1 antibody. A significant reduction in the number of aggregates versus monomeric α syn is present after AAVCSP α injection. Two-tailed Student's t test **p \leq 0.01; N=4 mice per group. **C** Median size of α syn aggregates showing no significant difference between EV- and CSP α -injected mice. Two-tailed Student's t test p \geq 0.01, N=4 mice. **D** Left panel, Cumulative histograms for α syn species distribution in EV- and CSP α -treated mice and difference between the two distributions. Right panel, Frequency values table of α syn species at the 36.5 nm intercept in EV- and CSP α -treated mice. Kolmogorov-Smirnov test **P<0.01, number of measured species EV: 4583, CSP α : 2574. **E** Representative *d*STORM images of α syn staining in CSP α or EV injected 1-120h α syn mouse striatum. Note the difference in size of α syn aggregates, (single α syn aggregates enlarged in boxed areas scale bar insets =200 nm).

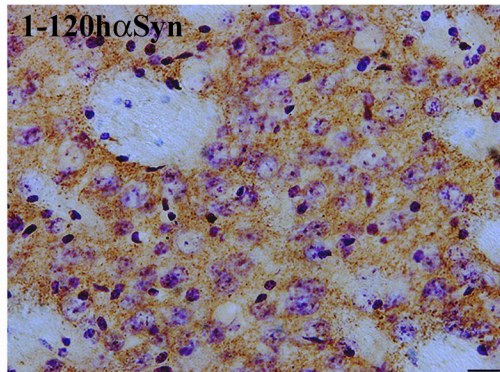
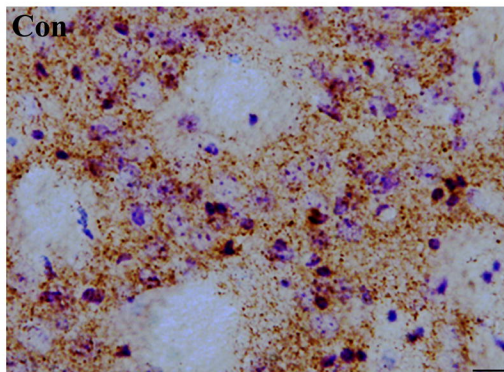
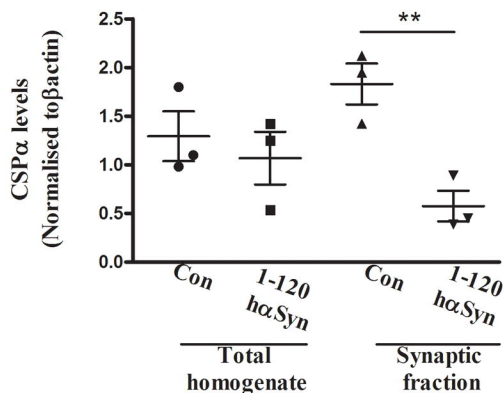
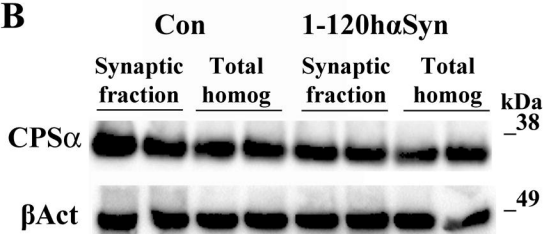
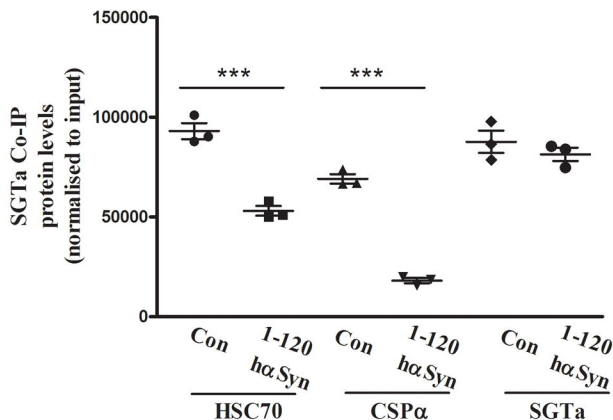
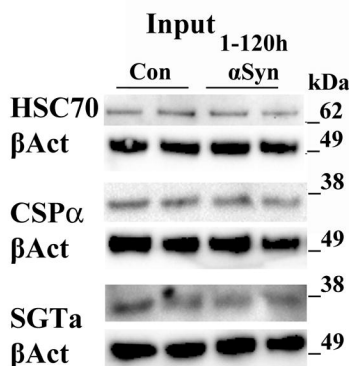
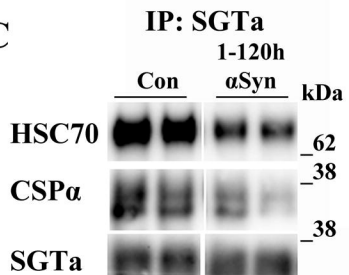
Supplementary Figure Legends

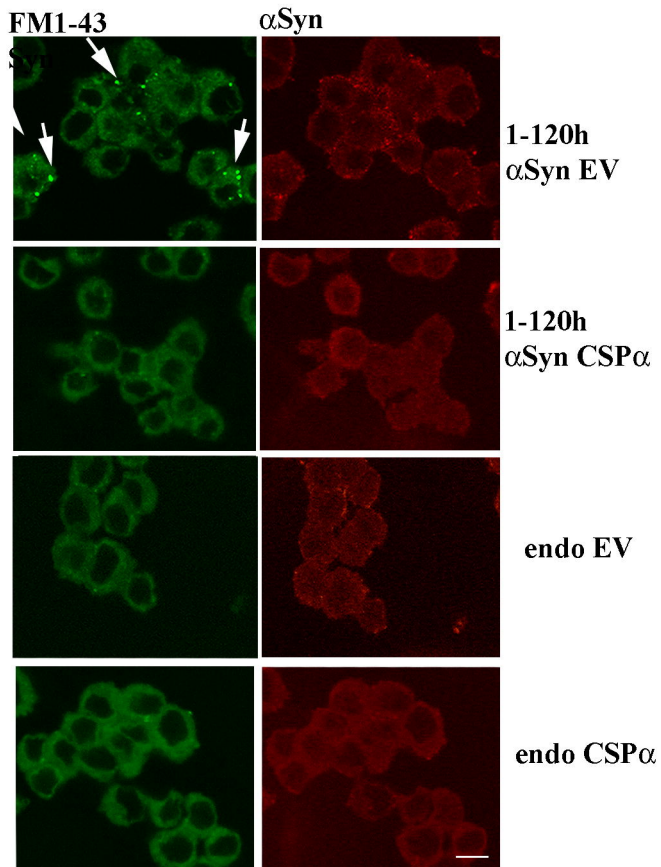
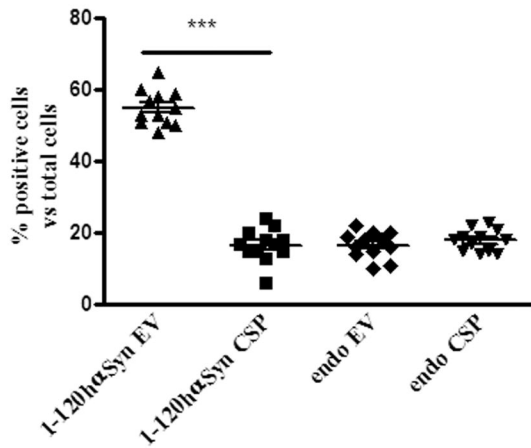
Supplementary Figure 1 HSC70, SGTa and VAMP2 expression in the striatum

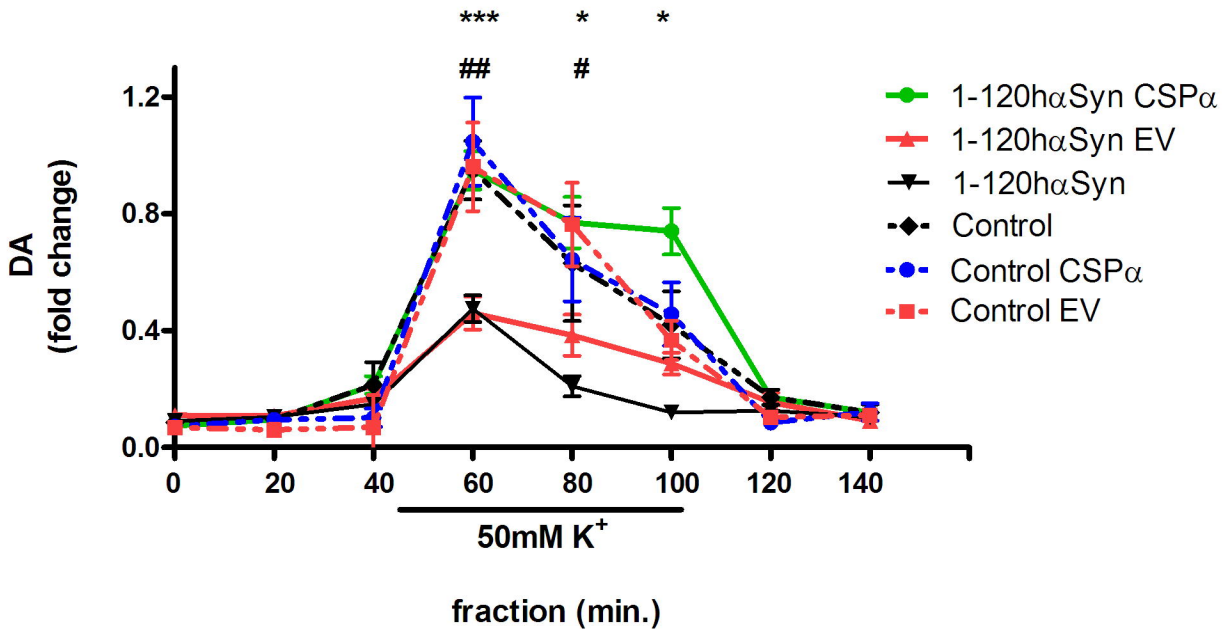
A (Left panel) Representative blots of synaptosomal fraction of HSC70, correspondent β Actin and normalised RI values (right panel, Control (Con) 0.29 ± 0.05 , 1-120h α Syn 0.42 ± 0.1). **B** (Left panel) Representative blots of synaptosomal fraction of SGTa, correspondent β Actin and normalised RI values (Right panel, Con 1.32 ± 0.06 , 1-120h α Syn 1.28 ± 0.08). **C** (Left panel) Representative blots of VAMP2 and correspondent β Actin in control and 1-120h α Syn mice from synaptic and total brain homogenates fractions. (Right panel) VAMP2 levels quantification (Synaptic fraction RI; Con 0.72 ± 0.07 , 1-120h α Syn, 0.65 ± 0.03 ; total homogenate; Con 0.25 ± 0.06 , 1-120h α Syn 0.34 ± 0.03) data are mean \pm SEM of N=3 mice per group, two-tailed Students t test for data in A and B, one-way ANOVA for data in C.

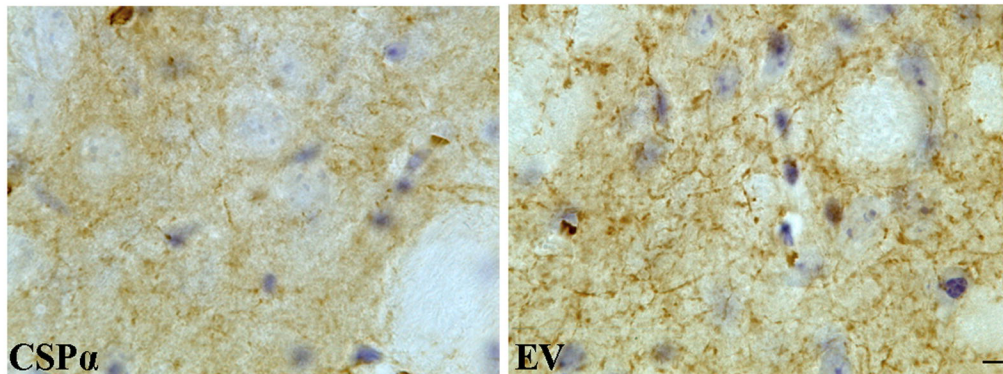
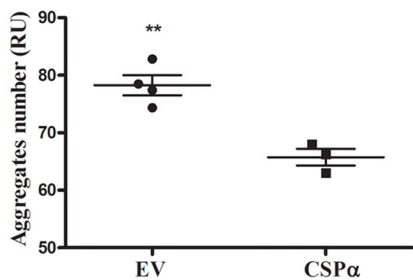
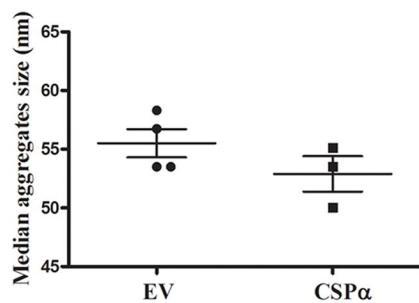
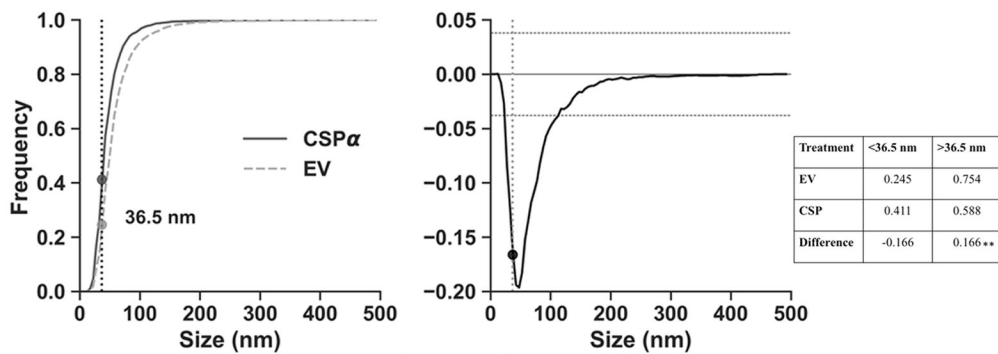
Supplementary Figure 2 Time course of CSP α expression following injection of AAVCSP α and AAVEV in mouse substantia nigra.

A Immunoblots of CSP α and β Actin in striatal extracts at 4, 6, 8 weeks post AAV injections in the SN. **B** Densitometry of CSP α levels at 4, 6 and 8 weeks post AAV injection. Data are mean \pm SEM of N=3 mice per group, Two-way ANOVA with Bonferroni's multiple comparisons test, 4wks ** P<0.01, 6 wks ***P<0.001, 8 wks ** P<0.01.

A**B****C**

A**B**



A**B****C****D****E**